Process for the preparation of one or more statins by fermentation

Description

Technical field

The present invention relates to process for the preparation of one or more statins by fermentation. The invention further relates to food product comprising one or more statins.

Background of the invention

Statins are compounds that are known to have a lowering effect on levels of low-density lipoprotein cholesterol (LDL-cholesterol) in the human blood. Elevated LDL-cholesterol levels (hypercholesterolemia) is directly related to increased risk of coronary heart disease. Statins inhibit the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the rate-determining step in the cholesterol biosynthesis.

Scientific research has confirmed the healthy properties of statins especially with respect to LDL blood-cholesterol and triglyceride levels lowering activities, both in animals and in humans (Li *et al.*, Nutrition Research 18, 71-81 (1998); Heber *et al.*, Am. J. Clin. Nutr. 69, 231-236 (1999)).

Early reports of the effect of statins were made in 1979. The Japanese scientist Endo isolated a metabolite from *Monascus* that reduced artificially induced hyperlipoproteinemia in rats (Endo, J. Antibiotics 32, 852-854, (1979)). These metabolites are known as monacolins. Monacolin is identical to the

cholesterol lowering pharmaceutical lovastatin. Lovastatin is sold by Merck co. under the tradename Mevacor. A derivative of lovastatin, simvastatin, is sold as a cholesterol-lowering drug under the name of Zocor. Other derivatives of lovastatin e.g. pravastatin, and mevastatin, are also sold as lipid lowering drugs against hypercholesterolemia. *Monascus*-extracts are sold in capsules in Japan as the dietary product Monacolin. The usual dose of the above statins is 20 mg/day, which results in at least 20% blood LDL-cholesterol lowering.

The production of statins is also reported in fermentation using fungi other than the above-mentioned *Monascus* species. It has been shown that statins can be produced by a variety of filamentous fungi, including *Monascus, Aspergillus, Penicillium, Pleurotus, Pythium, Hypomyces, Paelicilomyces, Eupenicillium, and Doratomyces*.

The preparation and purification of the statins used in pharmaceutical preparations involves many process-steps, in which ingredients are used that are not commonly used in the food industry. The many process steps increase costs compared to processes having less process steps. For these reasons the statins prepared for pharmaceutical use are not used in the foods industry.

As a food product, rice fermented with a red *Monascus* fungus (red rice) has been known and used for hundreds of years in China. Red rice was used and still is used in wine making, as a food-colouring agent and as drug in traditional Chinese medicine. We have found that most red rice available on the market contains no statins or statins in very low amounts. The Food and Drug Administration has concluded that red yeast rice

available in the market does not contain significant amounts of lovastatin (FDA, Docket No. 97-0441, Final Decision).

WO 99/23996 describes a composition for treating elevated serum cholesterol and/or triglycerides comprising a red rice product containing at least 0.05% lovastatin by weight.

Red rice powder capsules are sold as dietary supplements under the name of Cholestin by the firm Pharmanex. Pharmanex also sells a Cholestin bar containing red yeast rice (*Monascus purperus went*).

Red rice has an intensive red colour. Whereas the intensive red colour of red rice is an advantage when it is used as colouring agent, it is a disadvantage when it is used in food products. Due to the intense red colour of red-rice products, the foods prepared from red rice are coloured, depending on the amount of red-rice product added to the food product yellow, orange or red. The higher the amount of red rice added to the food, the more intense is the red colour of the food product. In the known food products a relatively large amount of red rice has to be added in order to add enough statins. This results in a red colour of the products that cannot be avoided.

In some food products the red rice colouring is undesirable. In particular in the western world, consumers are reluctant to use products of which the colour has changed from that they are used to. For example spreads, including margarine, butter, low fat spreads or salad oils are considered unacceptable by customers, when the colour of such a product is orange or red. However, at the same time these type of products have been found by us to be excellent vehicles of the daily intake of

amounts of statins sufficient to obtain a blood LDL-cholesterol lowering effect.

Summary of the invention

It is an object of the invention to provide a food product that has no undesired colouring due to the addition of statins. A further object of the invention is to increase the health effects of the known food products comprising statin. Another object is to provide a process for the preparation of a food product comprising statin, which involves less process steps than in the preparation of statins as a pharmaceutical drug. Another object is to provide such a process, which avoids the use of ingredients or process aids that are not commonly used in the food industry.

One or more of these objects are attained by a process for the preparation of one or more statins by fermentation, wherein a substrate is fermented with statins producing fungus, characterized in that substrate comprises more than 20% by weight of soy ingredients.

Further one or more of the above objects are attained by a food product comprising:

a) an amount of one or more statins

b) an amount of one or more compounds chosen from the group: polyunsaturated fatty acids, phytosterols, proteins, peptides, dietary fibers, including soluble fibers, polyphenols and saponins, wherein the food product has a Hue a value of less than 20, preferably less than 20, most preferably less than 0.

Preferably the amount of a) is 5-100 mg/kg and the amount of b) 1 wt. or higher. More preferably the amount of b) is 5 wt.% or higher.

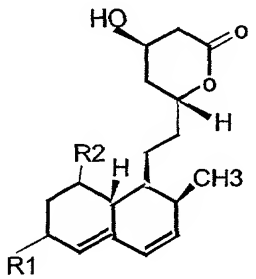
We have found that when the substrate for the *Monascus* fermentation is soybeans and/or soybean ingredients, the red colouring of the fermented product as in red rice fermentation is avoided, i.e. a non-coloured or only slightly coloured fermentation product is obtained. Further we have found that compounds having a positive health effect, which are present in soybeans are also present in the fermented product. These compounds include, but are not limited to polyunsaturated fatty acids, phytosterols, proteins, peptides, dietary fibers including soluble fibers, polyphenols and saponins. As a result of the presence of these compounds in the fermentation product, the food product according to the invention has increased health effects compared to the known food products comprising statin.

Detailed description of the invention

The following definitions will be used.

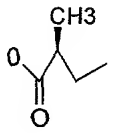
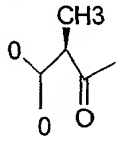
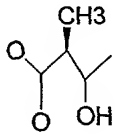
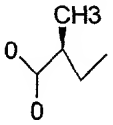
Statins are defined as substances having the structural formula, presented in formula (1).

(1)



In this structural formula, R1 and R2 can be any group. Preferred statins are those given in table 1.

Table 1: Preferred statins according to formula (1)

	R1	R2
monakolin K	CH3	
monakolin L	CH3	H
monakolin J	CH3	OH
monakolin X	CH3	
monakolin M	CH3	
compactin (ML-236B)	H	
ML-236-A	H	OH
NL-236-C	H	H

Polyphenols herein are polyphenols having plant origin. These include flavenoids, which include isoflavones. The polyphenols include isoflavones, stilbenes, lignans, coumestans and resorcyclic acid lactones. Examples of isoflavones are genistein, daidzein, equol, glycitein, biochanin A, coumestrol, maitaresinol, formononetin, O-desmethylengolesin, enterolactone and enterodiol. Preferred isoflavones according to the invention are genistein and daidzein and glycitein, which are present in soybeans.

Saponins are herein derived as β-D-glucopyranosiduronic acid derivates. Examples of saponins are Soya sapogenol A,B,C,D and E, Soyasaponin I, II and III, as described in Lebensmittel Lexikon, B.Behr's Verlag GmbH & Co. Hamburg, Bd.2, L-Z -3, 1993, pages 550-552.

Polyunsaturated fatty acid esters are defined as fatty acid esters having more than one unsaturated group in the fatty acid chain. Examples of polyunsaturated fatty acid esters are linoleic acid esters, linolenic acid esters, arachidonic acid esters.

Dietary fibers are herein a collective term for a variety of plant substances, that are resistant to digestion by the human gastrointestinal enzymes. Depending on their solubility, dietary fibers can be classified into insoluble (cellulose,

some hemicelluloses, lignins), and soluble (remainder of the hemicelluloses, gums, mucilages. Soybean colyledon fibers comprise both soluble and insoluble dietary fibers.

Phytosterols are herein defined as sterol compounds produced by plants, which are structually very similar to cholesterol except that they contain some substitutions at the C24 position on the sterol side chain. The phytosterols include 4-desmethylsterols, 4-monomethylsterols, 4,4'-dimethylsterols and mixtures thereof. Examples of such phytosterols are β-sitosterol, campesterol, stigmasterol. The term phytosterols herein also includes phytostanols, the saturated equivalents of phytosterols.

Polyphenols, polyunsaturated fatty acids, phytosterols, proteins, peptides, dietary fibers, and saponins will hereinafter collectively be referred to as soy actives.

Unless otherwise indicated, the amounts given will be expressed, in wt.% or weight parts per million (ppm), mg/kg or g/kg, relative to the total weight of the food product, unless otherwise indicated.

The amounts of statins given herein are the sum of the amounts of individual statins, as e.g. determined by chromatography, unless otherwise indicated.

The substrate is herein defined as total of compounds in the fermentation medium, without the solvent, for instance without water, in case a liquid, water based fermentation medium is used. In case no solvent is present the substrate equals the fermentation medium.

The protein amounts given herein are the sum of the amounts of individual proteins, unless otherwise indicated.

The amounts of soy actives are expressed as the sum (wt.% or ppm) of polyunsaturated fatty acids, phytosterols, proteins, peptides, dietary fibers including soluble fibers, polyphenols and saponins.

The food product has a Hue a* value of less than 20, preferably less than 20, most preferably less than 0. The Hue a* value is determined as described hereinafter in the examples.

Preferably a food product according to the invention does not include products especially suitable for the feeding of animals (feed).

Several food products may be prepared according to the invention, for example, spreads, soups, noodles, ice-cream, sauces, dressing, snacks, cereals, beverages, bread, biscuits, other bakery products, sweets, bars, chocolate, chewing gum, dairy products, dietetic products e.g. slimming products or meal replacers etc.

The statins and soy actives are present in the food product in an amount sufficient to obtain a blood LDL-cholesterol lowering effect if the food product is used according to the common needs of the consumer.

The skilled person will be able to adjust the percentage of statins and soy actives in the food product to get the above effect. The percentages will depend on the type of food product, since the food products are used in different serving

sizes. Moreover the pattern in a food product is consumed (servings per day and distribution over days) is dependent on the food product. Data about serving sizes may be found in the list published by the United States Food and Drug Administration (FDA) titled: "Reference Amounts customarily consumed per Eating Occasion".

Preferably in the food products according to the invention, the amount of statin is 5-500 mg/kg and the amount of soy actives is 1 wt. or higher. More preferably the amount of soy actives is 5 wt.% or higher or 10 wt% or higher. Most preferably the amount of soy actives is 20 wt.% or higher.

As an illustration table 2 indicates a number of products, which may be prepared according to the invention, and a typical serving size.

Table 2

Product	**Daily Serving**
Margarine	15 g
Meat product	50 g
Dressing	30 g
Sweet	10 g
Bar	75 g
Meal replacer drink	330 ml
Beverages	200 ml

Preferably the food product according to the invention comprises statin and non-glycosylated isoflavone. In soy beans and soy materials derived from soy, isoflavones are present substantially in the glycosylated form. Typically about 5 wt.% of the isoflavones is present in the non-glycosylated form. The most important glycosylated isoflavones are genistin, daidzin

and glycetin. The non-glycosylated forms are respectively genistein, daidzein and glycetein. Genistein, daidzein and glycetein have been reported to have advantageous health effects, including estrogenic and antioxidant properties.

We have found that due to the fermentation according to the invention the glycosylated isoflavones are converted into the corresponding non-glycosylated isoflavones, which are more benificial. For instance, the amount of genistein and daidzein is increased in the fermented soy compared to the non-fermented soy. Surprisingly this advantageous conversion occurs simultaneously with the production of statin.

The invention therefore further relates to a food product wherein the amount of statin is 5-500 mg/kg, and an amount of genistein and genistin, wherein the amount of genistein is 10-99 wt.%, preferably 15-99 wt.%, more preferably 20-95 wt.%, still more preferably 20-90 wt.%, most preferably 20-80 wt.% of the sum of the amounts of genistein and genistin.

The invention therefore further relates to a food product wherein the amount of statin is 5-500 mg/kg, and an amount of daidzein, wherein the amount of daidzein is 10-99 wt.%, preferably 15-99 wt.%, more preferably 20-95 wt.%, still more preferably 20-90 wt.%, most preferably 20-80 wt.% of the sum of the amounts of daidzein and daidzin.

The absolute amounts of genistein and daidzein may, for each food product, be adjusted by the skilled person to a desired level. This may for instance be done by selection of the soy material to be fermented from materials havaing a different isoflavone content, by adjustment of the fermentation conditions, such as fermentation time, and by selecting the

amount of fermented soy added to the food product. In such way the amount may be adjusted to a desired daily intake of the isoflavones, that could be for instance 50-80 mg/day for genistein. The preferred absolute level of genistein in the food products according to the invention, depends on the food type, and may be 50 mg/kg or more, more preferably 100 mg/kg or more, 200 mg/kg or more, 500 mg/kg or more and most preferably 200-5000 mg/kg. Also the absolute level of daidzein depends on the food type, and may be 50 mg/kg or more, more preferably 100 mg/kg or more, 200 mg/kg or more, 500 mg/kg or more and most preferably 200-5000 mg/kg.

Preferably the food product according to the invention is a spread, meat product, sauce, such as soy sauce, vinegar, soup, bakery good, beverage or bar. These products are preferred because the way in which they are consumed results in a more constant intake of statins and soy actives than in other food products. More preferred food products according to the invention are a spread, cereal bar, beverage or breakfast cereal.

The invention will now be further illustrated by the description of suitable embodiments of the more preferred food products. It belongs to the ability of the skilled person to use the teaching provided therewith to prepare other products of the invention.

- Spreads

Typically, spreads according to the invention are oil in water or water in oil emulsions, although also spreads, which are substantially fat free, are covered. Spreads are to include margarines and liquid cooking products. The spreads may be

spreadable and not pourable or may be pourable at the temperature of use e.g. 2-10 °C. Fat levels may vary in a wide range e.g. full fat margarines with 60-90 wt.% of fat, medium fat margarines with 30-60 wt.% of fat, low fat products with 10-30 wt.% of fat and very low or fat free margarines with 0 to 10 wt.% of fat.

The fat in the margarine or other spread may be any edible fat, often used are soybean oil, rapeseed oil, sunflower oil and palm oil. Fats may be used as such or in modified form e.g. hydrogenated, esterified, refined etc. Other suitable oils are well known in the art and may be selected as desired.

The pH of a margarine or spread may advantageously be from 5.0 to 6.5, though other pH's are possible.

Examples of spreads other than margarines are cheese spreads, sweet spreads, yoghurt spreads etc.

Optional further ingredients of spreads may be emulsifiers, colourants, vitamins, preservatives, emulsifiers, gums, thickeners etc. The balance of the product will normally be water.

A typical size for an average serving of margarine or other spreads is 15 grams. Preferred levels of statins in the margarine or spread are: 20-500 mg/kg statin, more preferred ranges are 50 to 250 mg/kg statin.

- <u>Beverages</u>

Preferred food products according to the invention are beverages, for example tea, fruit juice, soft drinks, meal-replacers, etc. Meal replacer drinks will be described in more detail herein below. It will be apparent that similar levels and compositions apply to other beverages comprising statins and soy actives.

A typical serving size of a beverage is taken to be 200 ml. Preferred levels of statins in a beverage are: 5-100 mg/kg, more preferably 10-80 mg/kg.

- Bars, including cereal bars

These products often comprise a matrix of edible material wherein the statin and soy actives can be incorporated. For example the matrix may be fat based (e.g. couverture or chocolate) or may be based on bakery products (bread, dough, cookies etc). Preferably the food product is a cereal bar, in which the matrix is based on agglomerated cereal particles (rice, grain, nuts, raisins, fruit particles).

The matrix material of a bar may be present in an amount of 60-95wt.% of the weight of the bar, preferably 70-90 wt.% most preferred 75-85 wt%.

Other ingredients in the cereal bar may be starch, sugar (e.g. 0-10wt%), sirups, honey, milk solids, salt (e.g. 0-5 wt.%) calcium carbonate, vitamins, flavouring and colouring.

The ingredients are usually mixed and cooked (e.g. by cooking-extruding) to produce the (cereal) bar.

A typical size for a bar could be from 20 to 200 g, generally from 40 to 100 g. Preferred level in such products would be: 25 to 500 mg/kg statin. More preferred range for this level is 50 to 300 mg/kg.

Further ingredients may be added to the product such as flavouring materials, vitamins, minerals etc.

Preparation of the food product

According to the invention a substrate, prepared from soybeans and/or ingredients thereof is fermented with a statin producing fungus and the fermentation product is used in the preparation of a food product. These steps will be illustrated below. In this illustration the statin producing fungus is *Monascus*.

Fermentation is conducted in known way. The fermentation is conducted in at least one fermentation vessel (fermenter) in which a medium comprising soybeans and/or ingredients thereof is present. The fermentation is started (inoculated) by adding a suspension of spores of the *Monascus* fungus (inoculum), which has been prepared by fermenting *Monascus* fungus on a separate medium. The fermentation may be executed batchwise or as a continuous process.

The fermentation involves the following steps, which are executed in the given order:

a) Preparation of the medium for the inoculum and the medium to be used in the fermenter

b) Sterilization of the media, fermenters and ancillary equipment
c) Production of inoculum
d) Addition of the inoculum to the medium comprising soybeans and/or ingredients thereof, in the fermenter.
e) Conducting the fermentation
f) Removal of the fermentation product from the fermenter

The fermentation product is used in the preparation of the food products according to the invention.

Optionally, before the fermentation product is used in the preparation of the food products, the following additional process steps may be executed:

g) Sterilization of the fermentation product
h) Drying of the fermentation product (or sterilized fermentation product)
i) One or more separation steps, for instance extraction, to separate statins and soy actives from *Monascus* biomass in the fermentation product

The medium used in the fermenter may be solid or liquid. Advantageously the medium is solid, most preferred the medium substantially consists of crushed whole soybeans, which have been soaked with water (e.g. 30 wt% water). In case the medium is liquid, usually water is present as major constituent of the medium.

Whole soybean are preferably used as a substrate for the fermentation. Typical composition of whole soybeans is 42% wt% protein, 20 wt% oil, 35% wt% total carbohydrates, 5 wt% ash and

5.5 wt% crude fiber (Kawamura, S., Tech. Bull. Faculty Agric., Kagawa Univ., 18, 117 (1967)).

Instead of whole soybeans, parts or ingredients of soybean may be used in the medium for the fermenter, for instance soy protein (including textured vegetable protein), soy milk, soy-flakes etc. Care has to be taken that the medium contains compounds that can provide a carbon source and a nitrogen source for growth of the *Monascus* fungus.

The *Monascus* fungus used according to the invention may be any *Monascus* fungus that produces statins. Preferably the fungus is chosen from the group of *Monascus ruber*
Most preferred is *Monascus ruber* F125 M1-4.

Strains F125 and F125 M1-4 are deposited at the Centraal Bureau voor Schimmelculturen (CBS) as no. CBS 109070 on 14.11.2000 and no. CBS 109269 on 23.01.2001.

These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty).

Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The medium will ordinarily be sterilized before fermentation, e.g. by heat treatment, like pasteurization.

The medium in the fermenter may contain other substances, which may aid the fermentation, for instance sugars, amino acids and vitamins.

The fermentation may be carried out in a manner, which can be determined by the skilled person on the basis of common general knowledge of fermentation technology. As illustration preferred embodiments are described hereunder.

The fermentation temperature may be important. The temperature is preferably in the range of 10 to 37, °C more preferably 20 to 30°C. We have found that at 37°C and higher the production of statins decreases.

Preferably during fermentation the medium is aerated, e.g. by stirring, shaking etc. Aeration may be carried out by blowing air through the fermentation medium. Preferably the air is wholly or partly saturated with water vapour in case solid state fermentation is used. This avoids drying out of the fermentation medium.

The relative levels of statins to soy actives will depend on the fermentation time. The fermentation time is therefore dependent on the desired amount of statins in the fermentation product. Preferred fermentation time is 1-60 days, more preferably 1-50 days, still more preferably 15-40 most preferably 20-30 days.

Prior to addition to the food product, the fermentation product may be subjected to a separation step, to separate statins and soy actives from *Monascus* biomass in the fermentation product. This separation may be done with known separation techniques, e.g. filtration or centrifugation.

The fermentation product may also be extracted and the extract may be used in the preparation of the food product. Preferred extraction agents are food-grade extraction agents. More preferred extraction agent is ethanol. Most preferred extraction agent is vegetable oil, e.g. soybean oil or sunflower oil.

The extraction may be done on the fermentation product. Alternatively the *Monascus* biomass may be separated from the fermentation product prior to extraction, e.g. by filtration. The *Monascus* biomass may be separately extracted and the resulting extract can also be used in the preparation of the food product.

Extracts may be used as such in the preparation of food products. Preferably extraction solvent may be removed from the extracts, e.g. by evaporation of the extraction solvent.

Advantageously edible oil may be used as extractant, the edible oil is preferably vegetable oil, such as for instance soy bean oil or sunflower oil. When the fermentation product is extracted with vegetable oil, it was found that the statin is effectively extracted and an oil phase containing substantially all statin is obtained. The resulting extract is very suitable to be used directly as a food ingredient.

Most advantageously the extractant, e.g. vegetable oil is added to the fermentation medium during fermentation. We have found that in the presence of an extractant, the production of statins during fermentation is considerably increased. It is possible to increase the amount of statin produced by at least a factor 10, more preferably at least a factor 40, compared to

fermentation without extractant, by the addition of vegetable oil during the fermentation. Preferably the extractant should not interfere with the fermentation, especially it should not be poisonous for the statins producing fungus. The amount of oil is preferably at least 5 wt.% oil (w/w on substrate), more preferably more than 10 wt.%, most preferably at least 20 wt.%, being present in the substrate during fermentation. Preferably the oil is edible oil, more preferably vegetable edible oil, such as for instance sunflower oil or soybean oil. Though animal and vegetable fat may be used these are less preferred for hearth health reasons.

The fermentation product (including extracts etc.) may directly be added to food product ingredients in the process of preparation of the food products according to the invention. It can be added to the other ingredients of the food product composition, or it may be added to part of the ingredients, before other ingredients are added. If more than one phase is present in the food product the fermentation product may be present in one or more of these phases. Preferably the fermentation product will substantially be present in an oil phase, if such oil phase is present.

The invention will be further illustrated in the examples.

Examples

Example 1

A. Preparation of *Monascus* strain F125 M1-4

Monascus ruber strain F125 was cultivated in malt water liquid medium at 30°C for 4 days. Of this culture, 1 ml was used as an inoculum for a Hybond-N filter (Amersham, UK) placed on a YE plate (4% glucose, 0.3% KH_2PO_4, 1.0% yeast extract (Difco), 1.5% agar). After 3 days incubation at 30°C, the spores were harvested by washing the filters with 10 ml physiological saline containing 0.1% Tween 80. The spores were filtered 4 times through Mira cloth filters to obtain a hyphae free spore suspension. This suspension was used for subsequent mutagenesis.

The spores were diluted to a concentration of 10^8 spore/ml then exposed to UV light at an intensity of 100 joules/m^2. The mutagenised spores were plated on Potato Dextrose Agar (Oxoid) and incubated for 3 weeks. One of the resulting colonies, which had a lighter colour than the others was selected and is herein defined as *Monascus* strain F125 M1-4.

B. Preparation of *Monascus* spores

Monascus f125M1-4 spores were prepared by harvesting *Monascus* mycelium from a PDA (potato dextrose agar) (oxoid) slope by washing with 5 ml physiological saline and incubating the mycelium in 150 ml malt water (oxoid) for 4 days at 30°C. The spores were harvested by filtration through a Mira cloth filter.

C. Fermentation

C.1. Preparation of an inoculum

A shake flask containing soybeans is inoculated with filamentous spores suspended in a physiological water solution containing 0.1 wt% Tween 80 (polyoxyethylene sorbitan fatty acid ester, available from ICI Specialty Chemicals™). This shake flask is incubated to let the mould grow. This resulted

in a *Monascus* spore suspension, which was adjusted by dilution to a concentration of $1*10^6$ spores/ml.

C.2. Preparation of fermentation medium

1 kg of soybeans is soaked in tap water (50°C) for 30 minutes. After soaking the beans are rinsed with cold tap water. Subsequently 50 g batches of the soaked beans (97% soybean) were brought in to 300 ml Erlenmeyer flasks.

C.3. Fermentation

The shake flasks were each inoculated with 1 ml of the prepared *Monascus* spore suspension and incubated for 30 days at 30 °C. A sample from the shake flask was taken every week to monitor the statin production.

After fermentation, about 600 g soybeans remained.

D. Statin analysis of fermentation product

The fermentation product samples are extracted in a 50 ml tube (Falcon) by adding 6 ml of a mixture of acetonitril, water and phosphoric acid (1:1:0.05, v/v/v). The mixture is blended with an Ultraturrax for 1 min. The mixture was then incubated at room temperature on a rollerbank for over 24 hours. Hereafter the samples were centrifuged and the supernatant liquid used for HPLC analysis. Samples were separated using HPLC analysis on a Shimadzu apparatus according to the method of Morovjan et al., J. chromatogr. A 763 (1997) 165-172. The system consists of the Shimadzu SCL-10A system controller, CTO-10AS column oven, LC-10AT vp pump system, RID-10A refraction index detector, SPD-M10A diode array detector and SIL-10AD autoinjector. For the chromatographic determination of statins a Waters NovaPak C18 (150x3.9 mm I.D., 4µm) column was used

operating at 25°C. The eluent was acetonitril-0.1% phosphoric acid (50:50,v/v) solution flowing at 1.5 ml/min. Runs were performed for 15 min. The detection was performed using a diode array detector from 190 nm up to 800 nm. The sum of the area of all peaks in the spectrum belonging to statins is measured. Comparison to a standard (Mevinolin, Sigma) allows the calculation of a statin content (expressed in mg/kg analysed product).

The analysis results in a statin content of 1200 mg/kg product.

E. Extraction of the fermentation product

The fermentation product was extracted with ethanol and acetonitril. The soybean extracts contained 0.0545 g statin/kg (ethanol extract) or 0.0978 g statin/kg (acetonitril extract) as determined by HPLC, as described under D.

F. Colour analysis of extract

When colours are classified, they can be broken down into the three primary elements. One is the Hue (colour) the other is Value (brightness) and the third is Chroma (Saturation like vivid colours or dull colours).

To enable anyone to tell anyone else exactly what colour they are talking about a common numerical code is used. This numerical code used is L*a*b*. When a colour is expressed in this system, Value becomes L*, while Hue and Chroma are expressed as a* and b* respectively. The L*a*b* was measured of different time samples during fermentation. The supernatant of the samples was filtered sterilized with a milipore 0.22µm filter. Of the clear liquid L*a*b* was measured with a UV 1601 spectofotometer of Shimatzu.

L*a*b* values of the soy extracts :

Soy fermented with *M. ruber M1-4*

L*= 90.2 ; a*=-4.8 ; b*=33.8

G. Preparation of a pourable margarine

An ethanol extract of the fermentation product was used for further processing. Ethanol was removed by means of rotary evaporator. The residue was used in a pourable margarine composition. A pourable margarine composition without fermentation product residue was used for comparison with respect to colour.

The composition of the pourable margarine is given in table 3.

Table 3. Composition of the pourable margarine of example 1.

Ingredient	**Amount (wt.% on total)**
Sunflower oil	79.6
Rapeseed oil hardened to a melting temperature of 70 °C	1.95
Lecithin (BOLEC MT)	0.18
Lecithin (Cetinol)	0.2
Potassium sorbate	0.0125
Water	18.0
Statins	0.00011

The colour of the pourable margarine was not different from a control with no statins extract, but 20 wt.% water.

Comparative example A

Example 1, steps B to G were repeated, but instead of *Monascus* strain F125 M1-4, a Monascus strain isolated from red rice (commercial product from China) was used.

The L*a*b* values of the extracts were determined according to example 1, step F. These values were:

L*= 90.2 ; a*=56.1; b*=63.9

Comparative example B

The procedure of example 1, steps B to G, was repeated, however in step C.2., instead of 1 kg of soybeans, 1 kg of rice (Parboiled rice, Oryza) was used. After fermentation about 500 g rice remained.

The L*a*b* values of the extracts were determined according to example 1, step F. These values were

L*= 80 ; a*=54; b*=49

Example 2

A spread having the composition as in table 4 was prepared.

Table 4: Composition of spread of example 2

Ingredient	Amount (wt% on total)
Sunflower oil	69.295
Fat blend	10.5
Beta carotene	0.125
Monoglygceride (Hymono 8903)	0.08
Water	13.33
Ethanol extract of example 1C	6.67
Total	100

The fat blend was a mixture of 65 wt.% mid fraction of palm oil stearin and 35 wt% palm kernel oil.

The spread was prepared by premixing the ingredients in a premix tank at 55°C in a premix tank and feeding the premix to a Votator with two scraped surface heat exchangers (A-units) operated at 800 rotations per minute (rpm) and one crystallizer (C-unit) operated at 200 rpm. The configuration was A-A-C.

The amount of statin in the spread was 74 mg/kg. The colour of the spread was not different from a control with no statins extract, but 20 wt.% water.

Example 3

Fermentation with oil addition

10 litre reactors were filled with 8 litres substrate (Glucose 20 g/l, glycerol 100 g/l, starch 20 g/l, NaNO3 2 g/l MgSO4 5 g/l) and set to an airflow of 6 volume change per minute (vvm). A pre-culture was prepared in YPD at 30°C for two days and used to inoculate the fermentor with a concentration of 1x10 7 spores/l *Monascus ruber* F125M1-4. The cultures were

incubated at 25°C and 200 rpm for 3 weeks. After 22 days, 400 ml soybean oil was added to the 10 litre reactor.

After 49 days the fermentation was stopped and the amount of statins in the water and the oil phase were measured. The results are shown in Table 5.

Table 5. Amount of statin (mg/l) in the different phases after 49 day of fermentation.

	Water phase	Oil phase	Total
Volume of the phase (l)	6.6	0.4	7
Statin (mg/l)	2.37	1420	83.4

At the end of the fermentation 97 wt.% of the statins were found in the oil phase. A big difference is seen between the statin levels in the oil and the water phase. The supplementation of oil increases the total amount of statin in the reactor. A control reactor without oil supplementation contained 1.8 mg/l statins, while the reactor with oil supplementation shows a total amount of 83.4 mg/l statins. The reactor with oil supplementation shows a 45 times higher statin content compared with the amount of statins in the fermentation without oil.

Addition of soybean oil to solid state fermentations also stimulated the production of statin.

Example 4

Steps A, B and D. were done according to example 1.

C. Fermentation

C.1. Preparation of an inoculum

A shake flask containing dehulled soybeans is inoculated with filamentous spores suspended in a physiological water solution containing 0.1 wt% Tween 80 (polyoxyethylene sorbitan fatty acid ester, available from ICI Specialty Chemicals™). This shake flask is incubated to let the mould grow. This results in a *Monascus* spore suspension, which was adjusted by dilution to a concentration of $1*10^6$ spores/ml.

C.2. Preparation of fermentation medium

1 kg of dehulled soybeans were soaked in tap water (50°C) for 30 minutes and subsequently air dried for two hours. Subsequently 50 g batches of the dried dehulled beans were brought in to 300 ml Erlenmeyer flasks.

C.3. Fermentation

The shake flasks were each inoculated with 1 ml of the prepared *Monascus* spore suspension and incubated for 30 days at 30 °C. A sample from the shake flask was taken every week to monitor the statin production.

After fermentation, about 600 g soybeans remained. The analysis according to step D results in a statin content of 2800 mg/kg product.

E. Isoflavone analysis of fermentation product

The isoflavone concentration was measured according to the HPLC method described in Franke A.A., et al. (1998): HPLC analysis of isoflavonoids and other phenolic agents from foods and human fluids; *Proceed.Soc.Exp.Biol.Med;* **217** (3), 274-280.

Two samples were tested. The first (comparative) sample was taken from non-fermented fermentation medium, as prepared in step C2 above. The second sample was of equal (solid) weight, but taken from the fermentation product. The results of the isoflavone measurement of these samples are given in table 3.

Table 3. Isoflavone concentration in fermented and non-fermented soy

Isoflavone	Isoflavone concentration (g/kg)	
	Fermentation medium (unfermented)	Fermentation product
Daidzin	1.107	0.304
Genistin	1.608	0.476
Daidzein	0.057	0.9
Genistein	0.085	0.494
Total	2.9	2.2

Table 3 shows that the amount of isoflavones in total in the fermentation product is slightly decreased compared to the non-fermented material, but surprisingly the amounts of genistein and daidzein are increased. The fermentation product contains substantial amounts of isoflavones in addition to the statin.